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Characterization of a Fluorescent Complex between Auramine O and Horse Liver Alcohol Dehydrogenase*

Richard H. Conrad,† James R. Heitz, and Ludwig Brand

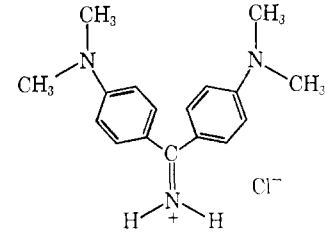
ABSTRACT: Auramine O exhibits an intense fluorescence in the presence of horse liver alcohol dehydrogenase but not with sixteen other proteins tested. The interaction between dye and protein has been studied by absorption and fluorescence spectroscopy, inhibition kinetics, and equilibrium dialysis. The results suggest two equal and independent dye binding sites with an association constant of 1.0×10^5 M^{-1}. The dye binding sites are distinct from the coenzyme and substrate binding sites as illustrated by the noncompetitive nature of the inhibition by auramine O. In the presence of oxidized nicotinamide-adenine dinucleotide (NAD^+), the number of dye binding sites is unchanged, the dye–protein association constant increases, and the dye fluorescence is quenched. Auramine O is displaced from the protein by addition of NAD^+ plus pyrazole. Energy transfer from intrinsic tryptophan to bound auramine O is observed, but no evidence has been obtained for transfer between dye molecules.

The binding of fluorescent dyes to specific sites on horse liver alcohol dehydrogenase has been under investigation in this laboratory with the aim of gaining insight into the nature of the active site and the function of the enzyme. Rose bengal and several *N*-arylaminonaphthalenesulfonates exhibit marked increases in fluorescence yield upon binding to liver alcohol dehydrogenase (Brand *et al.*, 1967; Conrad, 1968). Evidence was obtained that these dyes interact at the coenzyme binding regions of the protein.

It is of interest to note that the above dyes are anions. It is well documented that several buffer anions inhibit the catalytic activity of liver alcohol dehydrogenase (Sund and Theorell, 1963) and the binding of coenzymes (Li *et al.*, 1963). The binding of the fluorescent dyes could be due to their anionic character and/or their aromatic character. The fluorescence characteristics of the bound *N*-arylaminonaphthalenesulfonate dyes indicate that the binding sites are nonpolar regions on the enzyme (Turner and Brand, 1968).

This report describes the interaction of liver alcohol dehydrogenase with the *cationic* diphenylmethane dye, auramine O.

Auramine O does not fluoresce in water, ethanol, hexane, benzene, or dioxane. Intense visible fluorescence is observed in viscous solutions or when the dye is adsorbed to polyanions



such as DNA (Oster and Nishijima, 1964). The experiments to be described in this paper characterize a specific fluorescent complex between auramine O and liver alcohol dehydrogenase.

Materials and Instrumentation

Crystalline horse liver alcohol dehydrogenase, (EC 1.1.1.1) was obtained from C. F. Boehringer, Mannheim, West Germany, and dialyzed against 0.1 M sodium phosphate (pH 7.4) as previously described (Brand *et al.*, 1967). The molar extinction coefficient of liver alcohol dehydrogenase at 280 mμ was determined by the method of Sund and Theorell (1963). It was found to be within 2% of 3.54×10^4, the value reported by these authors. Other enzymes were obtained from the following sources: yeast alcohol dehydrogenase, bovine serum albumin, and urease from the Sigma Chemical Co., St. Louis, Mo.; beef liver glutamic dehydrogenase, rabbit muscle glycerol 1-phosphate dehydrogenase, rabbit muscle enolase, rabbit muscle aldolase, rabbit muscle triose phosphate isomerase, and α-chymotrypsin from C. F. Boehringer, Mannheim, West Germany; and rabbit muscle lactic dehydrogenase, egg-white lysozyme,

* Contribution No. 584 from the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218. *Received December 2, 1969.* Supported by Grant GM 11632 from the National Institutes of Health.

† Portions of the material presented here were taken from a thesis submitted by R. H. C. to The Johns Hopkins University in partial fulfillment of the requirements for the Ph.D. degree. Present address: Institute of Molecular Biophysics, Florida State University, Tallahassee, Fla. 32306.

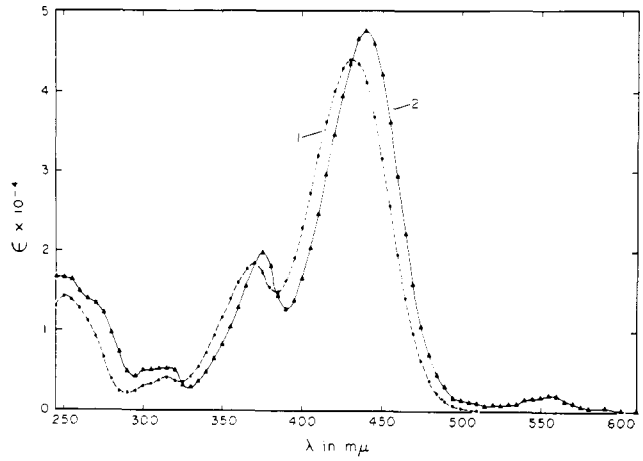


FIGURE 1: Absorption spectra of free auramine O (●--●) and auramine O bound to liver alcohol dehydrogenase (▲—▲). Free: 2.2×10^{-5} M auramine O in 0.1 M sodium phosphate at pH 7.4 read *vs.* sodium phosphate. Bound: 8.8×10^{-4} M liver alcohol dehydrogenase and 1.8×10^{-6} M auramine O in 0.1 M sodium phosphate at pH 7.4 read *vs.* 8.8×10^{-4} M liver alcohol dehydrogenase in phosphate buffer. It is assumed that the absorption of liver alcohol dehydrogenase does not change upon binding of the dye. ϵ = molar extinction coefficient (1 M^{-1} cm^{-1}).

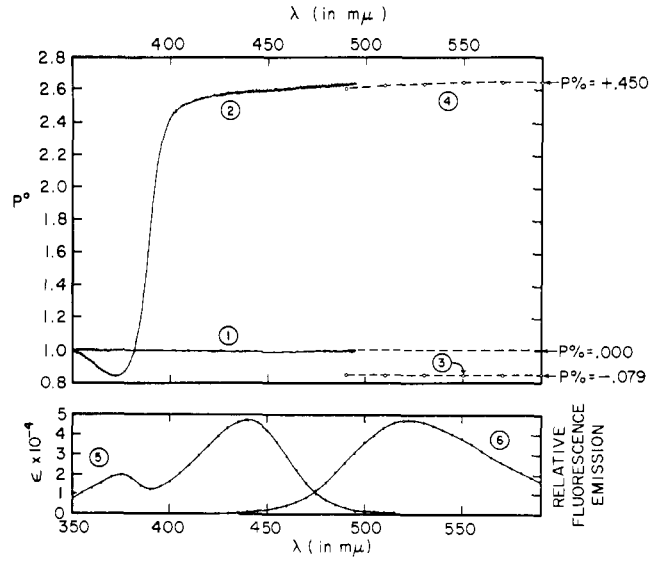


FIGURE 2: Polarization and fluorescence spectra of auramine O bound to liver alcohol dehydrogenase. Cuvets contained 4.4×10^{-6} M auramine O and 5.7×10^{-6} M liver alcohol dehydrogenase in 0.1 M sodium phosphate buffer (pH 7.4). Curve 1, base line ($P^0 = 1$); curve 2, P^0 of emission at 515 mμ as a function of exciting wavelength; curve 3, P^0 as a function of emission wavelength with excitation at 375 mμ; curve 4, P^0 *vs.* emission wavelength with excitation at 450 mμ. Curves 1 and 2 are direct instrumental tracings. $P^0 = I_V/I_H$ with excitation by vertically polarized light. [$P\% = (I_V - I_H)/(I_V + I_H)$.] The base line was obtained by excitation with horizontally polarized light. Curve 5, excitation spectrum of bound auramine O. Curve 6, corrected fluorescence emission spectrum of bound auramine O.

bovine trypsin, pancreatic ribonuclease, and pancreatic deoxyribonuclease-1 from Worthington, Freehold, N. J. The myosin was a gift from Dr. R. Josephs and *Drosophila* alcohol dehydrogenase was a gift from Dr. W. Sofer.

Pyrazole (mp 66–67°) was obtained from the Aldrich Chemical Co., Cedar Knolls, N. J., and used without further purification. NAD^+ was obtained from the Sigma Chemical Co. Fresh solutions were made up daily in glass-distilled water and stored at 4°. Pharmco USP 200 proof ethanol was obtained from Publicker Industries, Inc., Philadelphia, Pa., and used without further purification. Auramine O was obtained from Allied Chemicals, N. Y., and was recrystallized slowly from 0.02 M NaCl, the temperature being kept below 30° to avoid hydrolysis (Holmes and Darling, 1924). The large gold plates were dried *in vacuo* over P_2O_5 for 36 hr and stored in the dark. The following analysis was obtained for this material by Galbraith Analytical Laboratories, Knoxville, Tenn. *Anal.* Calcd for $[(CH_3)_2NC_6H_4]_2CNH_2Cl \cdot H_2O$: C, 63.44; H, 7.52; Cl, 11.02; N, 13.06; O, 4.96. Found: C, 63.27; H, 7.47; Cl, 10.87; N, 13.23; O, 5.16. It was established that auramine O in 0.1 M sodium phosphate buffer at pH 7.4 obeys Beer's law in the concentration range 2.21×10^{-6} to 2.21×10^{-3} M. The molar extinction coefficient at 430 mμ in this buffer was found to be 4.41×10^4 l. M^{-1} $cm^{-1} \pm 2\%$. The solubility is 0.74% in water and 4.49% in ethanol (Conn, 1961). The dye exists as a cation at neutral pH.

Fluorescence spectra were obtained with a ratio fluorometer constructed in this laboratory (Witholt and Brand, 1968), and are corrected. Optical densities were less than 0.08 at all excitation and emission wavelengths. Fluorescence kinetic measurements utilized an Aminco-Keirs spectrofluorometer. Absorption spectra were obtained on a Cary Model 14 spectrophotometer through the kindness of Professor A. Nason. All measurements were carried out in 0.1 M sodium phosphate buffer (pH 7.4) at room temperature.

Results

Evidence for a Fluorescent Complex. A solution of auramine O in 0.1 M sodium phosphate buffer has no detectable fluorescence. Any emission between 300 and 650 mμ with a quantum yield greater than 5×10^{-5} would have been observed. Upon addition of even very low concentrations of liver alcohol dehydrogenase, an intense fluorescence with emission maximum at 523 mμ is observed. This emission is excited by ultraviolet and blue light and has a quantum yield of about 0.05.

In contrast to the striking auramine O fluorescence observed with liver alcohol dehydrogenase, no fluorescence was observed in the presence of any of sixteen other proteins tested. These included the following: yeast alcohol dehydrogenase, *Drosophila* alcohol dehydrogenase, rabbit muscle lactic dehydrogenase, beef liver glutamic dehydrogenase, rabbit muscle glycerol 1-phosphate dehydrogenase, bovine serum albumin, rabbit muscle enolase, rabbit muscle aldolase, rabbit muscle triose phosphate isomerase, urease, egg-white lysozyme, α-chymotrypsin, trypsin, pancreatic ribonuclease, deoxyribonuclease, and myosin. Moreover, liver alcohol dehydrogenase is (with the possible exception of yeast alcohol dehydrogenase) the only protein of 21 tested by equilibrium dialysis in which binding can be demonstrated (J. R. Heitz and L. Brand, preliminary results). The proteins were not extensively dialyzed.

The absorption spectra of auramine O in phosphate buffer and in the presence of excess liver alcohol dehydrogenase are

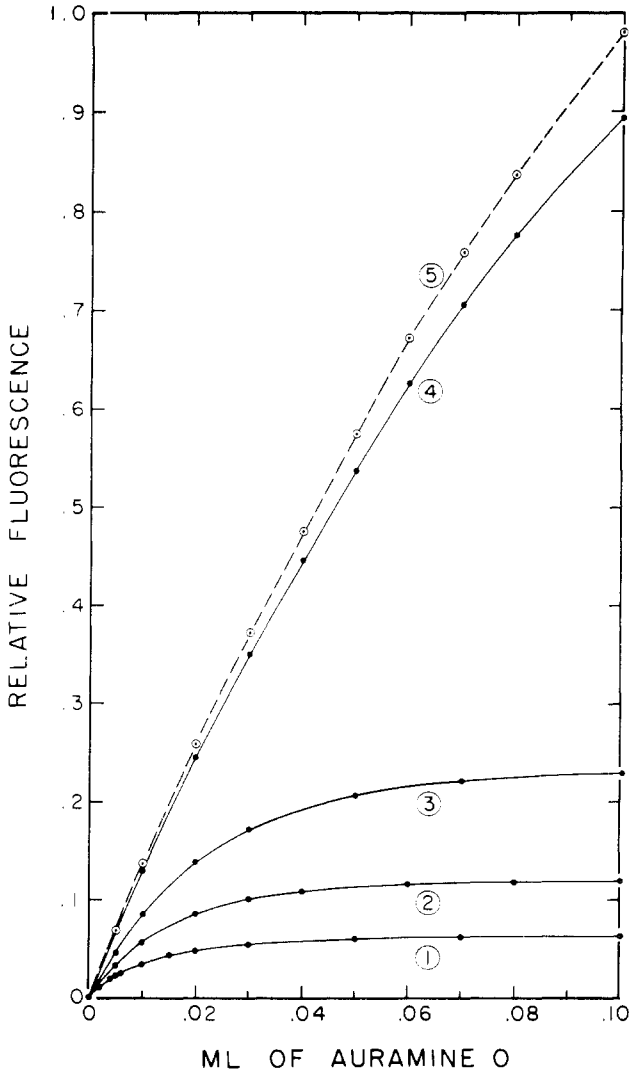


FIGURE 3: Fluorescence titration of liver alcohol dehydrogenase with auramine O. Excitation was at 500 mμ, emission at 544 mμ. The initial volume for each titration was 2.9 ml and liver alcohol dehydrogenase concentrations were: curve 1, 3.1×10^{-6} M; curve 2, 6.0×10^{-6} M; curve 3, 1.16×10^{-5} M; curve 4, 8.60×10^{-5} M. The concentration of the stock auramine O was 2.22×10^{-3} M. Titrations were carried out in 0.1 M sodium phosphate (pH 7.4). Curve 5 represents the theoretical fluorescence expected if all the auramine O were bound.

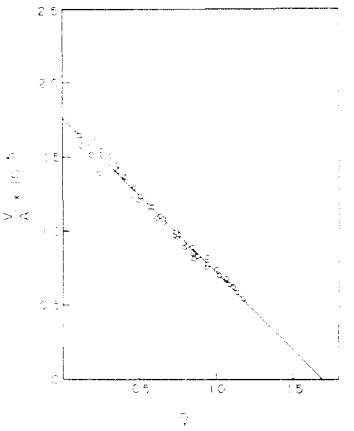


FIGURE 4: Scatchard plot of auramine O binding to liver alcohol dehydrogenase. In this case the titration data was obtained with excitation at 340 mμ and emission at 520 mμ at protein concentrations of 3.7×10^{-6}, 7.5×10^{-6}, 1.49×10^{-5}, and 1.86×10^{-5} M. The dye concentration at the end of the titrations was 2.5×10^{-5} M.

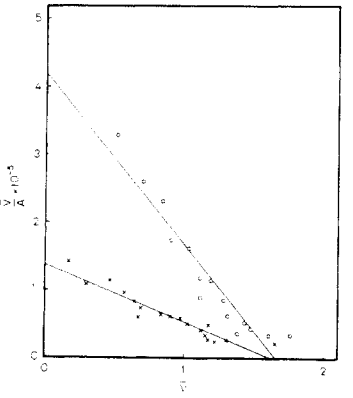


FIGURE 5: The effect of NAD^- on auramine O binding to liver alcohol dehydrogenase as measured by equilibrium dialysis. (X-X-X) Dialysis cells contained 5.4×10^{-6} M liver alcohol dehydrogenase and 0 to 6.9×10^{-5} M auramine O in 0.1 M sodium phosphate (pH 7.4). (O—O—O) Dialysis cells contained 5.4×10^{-6} M liver alcohol dehydrogenase, 6.9×10^{-5} M NAD^+, and 0 to 5.89×10^{-5} M auramine O in 0.1 M sodium phosphate (pH 7.4). Equilibrium was achieved within 18 hr at room temperature. Auramine O was assayed spectrophotometrically inside *and* outside the membrane at 430 mμ, the isosbestic point between free and bound dye.

shown in Figure 1. There is a significant spectral shift in the presence of enzyme suggesting binding of the dye.

In order to determine if the fluorescence of auramine O in the presence of liver alcohol dehydrogenase is actually due to a binding to the enzyme, measurements of the polarization of auramine O emission were carried out. Figure 2 contains the excitation and emission spectra of the fluorescence and the fluorescence polarization of auramine O in the presence of liver alcohol dehydrogenase. The fluorescence excitation spectrum is identical with the absorption spectrum of auramine O bound to the protein. Curves 1 and 2 are direct instrument tracings of the base line and the principal polarization spectra, respectively. Curve 2 shows a trough at 372 mμ corresponding to the lower excitation peak at 375 mμ, and has a plateau of high polarization through the 439-mμ excitation transition. The polarization is independent of the emission wavelength with excitation at 375 mμ (curve 3) or with excitation at 439 mμ (curve 4).

The excited state lifetime of auramine O bound to liver alcohol dehydrogenase was measured with a TRW instrument (Chen *et al.*, 1967) through the kindness of Dr. Chen and was found to be 3 ± 1 nsec. With this lifetime, an auramine O molecule freely rotating in aqueous solution would have a degree of polarization less than 1.2. The high polarization of 2.6 (maximum possible $P^0 = 3$) actually observed indicates that the dye is bound rather firmly to the protein.

From the data displayed in Figure 2, it appears that the 375- and 439-mμ bands represent separate and nonparallel electronic transitions with the emission emanating from the

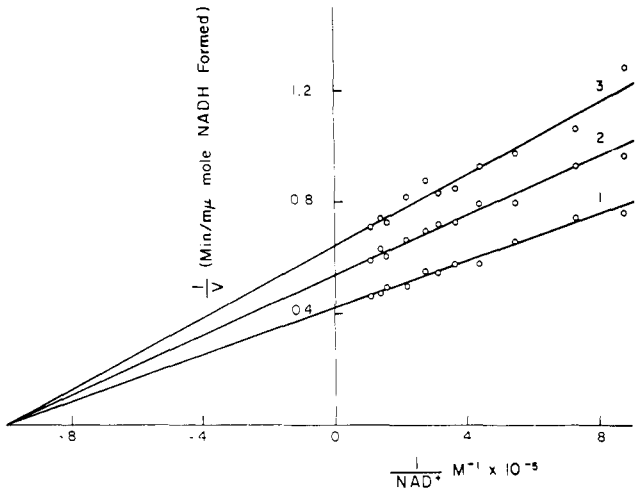


FIGURE 6: Lineweaver–Burk plot of auramine O inhibition of liver alcohol dehydrogenase. Formation of NADH was followed fluorometrically with an Aminco-Bowman spectrophotofluorometer; excitation at 350 mμ and emission at 460 mμ. Reaction mixtures contained 5.77×10^{-3} M ethanol, from 9.20×10^{-5} to 9.20×10^{-4} M NAD^+, and (curve 1) no inhibitor, (curve 2) 1.78×10^{-6} M auramine O, (curve 3) 3.57×10^{-6} M auramine O in a total volume of 3 ml of 0.1 M sodium phosphate at pH 7.4. The reactions were initiated by addition of 8.8×10^{-12} mole of enzyme.

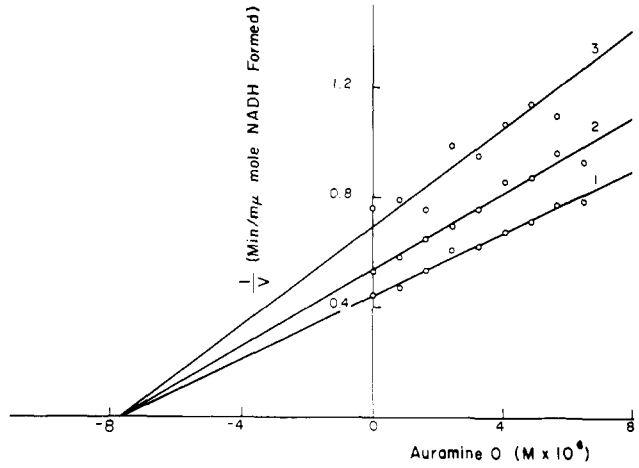


FIGURE 7: Dixon plot of auramine O inhibition of liver alcohol dehydrogenase. Reaction mixtures contained 5.77×10^{-3} M ethanol, from 0 to 6.5×10^{-6} M auramine O, and (curve 1) 1.38×10^{-4} M NAD^+, (curve 2) 3.68×10^{-5} M NAD^+, (curve 3) 1.38×10^{-5} M NAD^+. Other conditions as described in the legend to Figure 6.

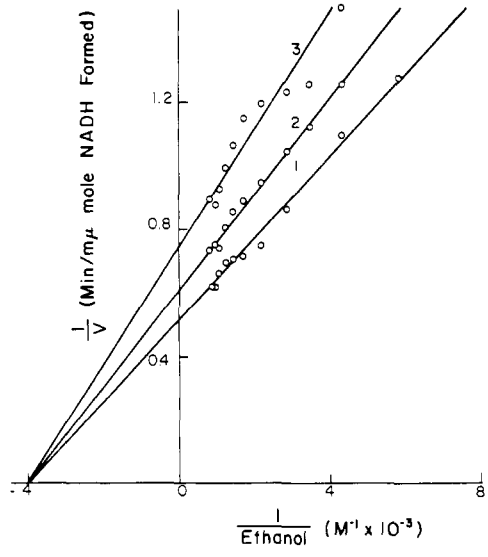


FIGURE 8: Noncompetitive inhibition of liver alcohol dehydrogenase by auramine O. Reaction mixtures contained 1.38×10^{-4} M NAD^+, 1.15×10^{-4} to 1.15×10^{-3} M ethanol, and (curve 1) no inhibitor, (curve 2) 1.22×10^{-6} M auramine O, (curve 3) 2.03×10^{-6} M auramine O. Other conditions as described in the legend to Figure 6.

transition at 439 mμ. The spectra are consistent with there being only one species of bound auramine O. Further evidence for such binding is provided by experiments to be described later which reveal that efficient transfer of excitation energy occurs from liver alcohol dehydrogenase to auramine O.

In order to ascertain if the binding of auramine O to liver alcohol dehydrogenase is noncovalent and completely reversible, a concentrated solution of the enzyme and dye was stored at 5° for 4 days and then applied to a Sephadex G-25 column. Elution with phosphate buffer cleanly resolved the mixture into two fractions. The first fraction contained only the protein and the second only auramine O. Neither fraction alone exhibited any auramine O fluorescence. When the two fractions were recombined, auramine O fluorescence identical with that shown in Figure 2 was obtained.

Quantitative Characterization of Auramine O Binding to Liver Alcohol Dehydrogenase. A fluorescence titration of liver alcohol dehydrogenase with auramine O is shown in Figure 3. Each curve represents a different protein concentration. The titration data are plotted according to the method of Scatchard (1949). In Figure 4 $\bar{\nu}$ equals bound dye per total protein concentration and [A] equals free auramine O. The straight line obtained is consistent with 1.7 equal and independent binding sites with an association constant of 1.1×10^5 M^{-1}. The number of binding sites may be compared with the well-known stoichiometry of two coenzyme binding sites for liver alcohol dehydrogenase (Theorell and Bonnichsen, 1951).

The interaction of auramine O with liver alcohol dehydrogenase was also investigated by means of equilibrium dialysis. The results shown in Figure 5 (lower curve) are in good agreement with the fluorescence titration giving 1.6 binding sites with an association constant of 0.85×10^5 M^{-1}.

Kinetic experiments were carried out to determine whether auramine O inhibits the catalytic activity of liver alcohol dehydrogenase. Initial rate measurements were obtained by observing the fluorescence increase due to the formation of NADH. (These measurements were corrected for the small effect of auramine O extinction on the observed NADH fluorescence.) Figure 6 shows a Lineweaver–Burk (1934) plot of auramine O inhibition with NAD^+ as the variable substrate. The inhibition is noncompetitive with NAD^+ and yields a K_I of 6.9×10^{-6} M. Figure 7 is a Dixon (1953) plot of auramine O inhibition of liver alcohol dehydrogenase. The lines intersect on the x axis consistent with noncompetitive inhibition. The value of K_I (7.7×10^{-6} M) obtained from this

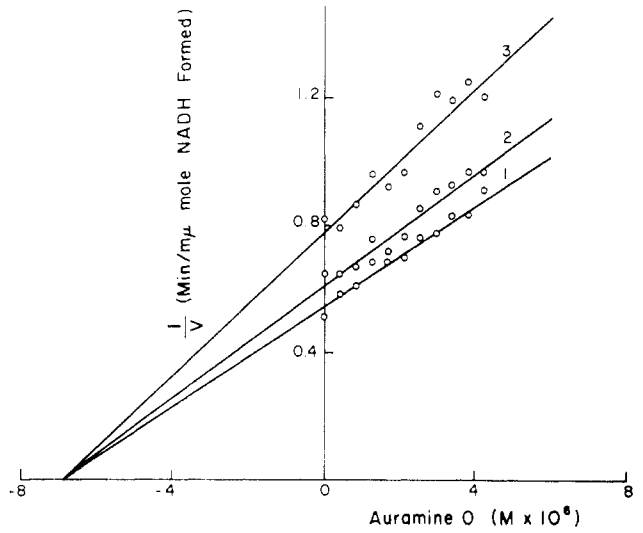


FIGURE 9: Inhibition of liver alcohol dehydrogenase as a function of auramine O concentration. Reaction mixtures contained 1.38×10^{-4} M NAD^+, auramine O varied from 0 to 4.23×10^{-6} M and (curve 1) 5.77×10^{-4} M ethanol, (curve 2) 1.15×10^{-3} M ethanol, (curve 3) 1.73×10^{-3} M ethanol. Other conditions as described in the legend to Figure 6.

data is in good agreement with that obtained by varying NAD^+ concentration.

Since auramine O binding is noncompetitive with NAD^+, it was of interest to determine if the dye was binding at the ethanol binding site. Figure 8 is a double-reciprocal plot of auramine O inhibition with ethanol as the variable substrate. The K_I obtained from this plot is 5.4×10^{-6} M and a K_I of 6.5×10^{-6} M is obtained from the Dixon plot shown in Figure 9. It is clear from Figures 8 and 9 that auramine O is a noncompetitive inhibitor with respect to ethanol.

These kinetic measurements give a K_M value of 1.0×10^{-5} M for NAD^+ and a K_M value of 2.5×10^{-4} M for ethanol in agreement with the values of Theorell *et al.* (1955). The average K_A of auramine O determined from the four kinetic inhibition studies is 1.5×10^{5} M^{-1}.

The effect of NAD^+ on the auramine O fluorescence of the dye–enzyme complex is shown in Figure 10. Strong fluorescence quenching is observed upon addition of NAD^+. The

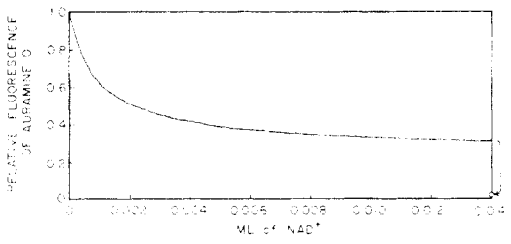


FIGURE 10: Fluorescence titration of the auramine O–liver alcohol dehydrogenase complex with NAD^+, and the effect of pyrazole. The cuvet initially contained 2.41×10^{-5} M auramine O and 3.98×10^{-6} M liver alcohol dehydrogenase. NAD^+ (0.047 M) was added to a concentration of 2.53×10^{-4} M, whereupon the solution was made 2.7×10^{-3} M in pyrazole (arrow). Excitation was at 480 mμ and emission was at 530 mμ.

TABLE I: Polarization of Auramine O.[a]

$\bar{\nu}$	P^0
0.05	2.59
0.20	2.58
0.40	2.61
0.55	2.63
1.0	2.63
1.4	2.64
1.6	2.67
1.7	2.67

[a] Excitation was with vertically polarized light at 490 mμ and the polarization of the emission was observed at 580 mμ. Values of $\bar{\nu}$ at each concentration of dye were calculated assuming two binding sites and a K_A of 1.1×10^{5} M^{-1}.

decrease in fluorescence is too great to be due to a simple competition between auramine O and NAD^+ for the same binding sites on the protein. At the end of the titration an excess of pyrazole was added to the cuvet. As indicated by the arrow, essentially all the auramine O fluorescence was lost.

The effect of NAD^+ on the interaction of auramine O with liver alcohol dehydrogenase as measured by equilibrium dialysis is shown in the upper curve of Figure 5. The coenzyme does not release auramine O from the enzyme but instead increases the auramine O–enzyme association constant to 2.5×10^{5} M^{-1} and quenches the fluorescence of the bound auramine O. The stoichiometry remains unchanged. Other equilibrium dialysis experiments (J. R. Heitz J. H. Easter, and L. Brand to be published) have shown that auramine O does *not* bind to liver alcohol dehydrogenase in the presence of *both* NAD^+ and pyrazole. Absorption spectra have not revealed any indications of complexing between auramine O and NAD^+ and/or pyrazole in the absence of liver alcohol dehydrogenase.

Fluorescence Characteristics. It is of considerable interest that the fluorescence polarization of auramine O bound to liver alcohol dehydrogenase does not decrease with increasing saturation of the enzyme as indicated in Table I. Such a decrease in polarization would be expected if energy transfer occurred between two dye molecules bound to the same protein molecule in nonparallel orientation (Weber, 1966).

When liver alcohol dehydrogenase is excited by light, some of the energy absorbed by the aromatic amino acids appears to be transferred to bound auramine O. Evidence for this transfer is contained in Figure 11. Curve 4 is the molar extinction spectrum of liver alcohol dehydrogenase. Curve 3 is the molar extinction spectrum of auramine O when bound to liver alcohol dehydrogenase (taken from Figure 1). Curve 1 is the molar extinction spectrum of an auramine O–liver alcohol dehydrogenase complex with a 1:1 molar ratio of dye to enzyme (calculated by summing curves 3 and 4). Curve 2 is an excitation spectrum of bound auramine O fluorescence with emission measured at 530 mμ. It is normalized to curves 1 and 3 at 375 mμ. The $\bar{\nu}$ in this case is 0.2 and therefore only 10% of the emission is due to complexes with a 2:1 dye to enzyme ratio. The excitation in the 260–290-mμ region (curve 2) may be contrasted with the relatively low absorption

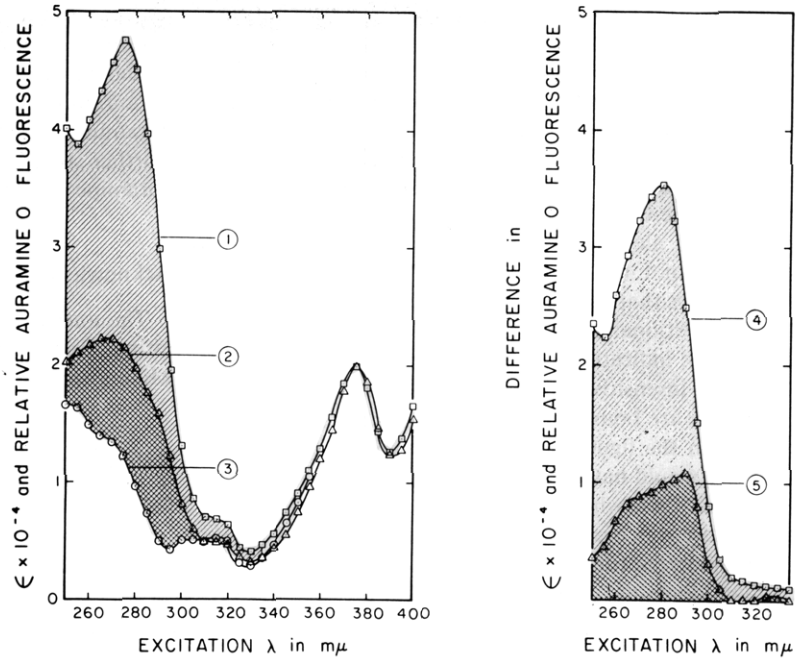


FIGURE 11: Energy transfer from liver alcohol dehydrogenase to bound auramine O. Curve 4 is the molar extinction spectrum of liver alcohol dehydrogenase. Curve 3 is the molar extinction spectrum of auramine O when bound to liver alcohol dehydrogenase (taken from Figure 1). Curve 1 is the molar extinction spectrum of an auramine O–liver alcohol dehydrogenase complex with a 1:1 molar ratio of dye to enzyme (calculated by summing curves 3 and 4). Curve 2 is an excitation spectrum of bound auramine O fluorescence with emission measured at 530 mμ. It is normalized to curves 1 and 3 at 375 mμ. Curve 5 = curve 2 minus curve 3. $\bar{\nu} = 0.2$.

in this region due to auramine O (curve 3). The increased fluorescence excitation, or the difference between curves 2 and 3, is represented by curve 5. The shape of this difference curve approximates the excitation spectrum of liver alcohol dehydrogenase, indicating that it is probably due to a transfer of excited state energy from liver alcohol dehydrogenase to bound auramine O which is qualitatively similar to the tryptophan–1-dimethylaminonaphthalene-5-sulfonate transfer described by Conrad and Brand (1968). The number of quanta transferred to each auramine O per quanta absorbed by bound liver alcohol dehydrogenase is the transfer efficiency. This is equal to the ratio of the areas under curves 5 and 4, which is roughly 0.3.

Preliminary measurements on complexes with a 2:1 dye to enzyme ratio have indicated that the transfer efficiency is decreased to about 0.2.

An expected corollary of the above transfer, a decrease in protein fluorescence with increasing saturation of the enzyme, is observed as indicated in Figure 12.

Discussion

The Number of Binding Sites. It is clear that auramine O binds reversibly to liver alcohol dehydrogenase and has little rotational flexibility independent of the protein. In spite of the fact that we consistently find a stoichiometry slightly less than two, it is most likely that there is one dye binding site for each coenzyme binding site.

There are several possible explanations for the low stoichiometry obtained. A steroid alcohol dehydrogenase is known to be present in liver (Pietruszko *et al.*, 1969). These workers report that liver alcohol dehydrogenase, from the same commercial source used in our studies, contained less than 4% of the steroid dehydrogenase. We find less than 1% by polyacrylamide gel electrophoresis. Therefore it is unlikely that this could explain the low number of binding sites.

We have observed that auramine O does not bind to acid denatured liver alcohol dehydrogenase with the formation of a fluorescent complex. If the native enzyme were in equilibrium with a denatured or partially unfolded form which did not bind auramine O but was still capable of binding NAD^+–pyrazole, the slightly low values of n obtained for auramine O would be accounted for.

Fluorescence Polarization. A measurable amount of energy transfer should take place between two auramine O molecules bound to the same liver alcohol dehydrogenase unless the chromophores are more than about 40 Å apart and/or have their transition dipoles oriented nearly perpendicular to each other. This was concluded from calculations similar to those described in Conrad and Brand (1968) which utilize the quantum yield of auramine O and an overlap integral (Förster, 1947) of 9.0×10^{-12} cm^6 mole^{-1}. The fact that no depolarization of auramine O fluorescence occurs when more than one dye molecule is bound per liver alcohol

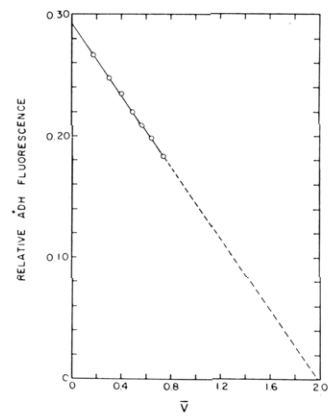


FIGURE 12: Quenching of liver alcohol dehydrogenase fluorescence as a function of $\bar{\nu}$, the average number of auramine O molecules bound. A total of two binding sites was assumed in calculating $\bar{\nu}$ from auramine O fluorescence.

dehydrogenase (as shown in Table I) indicates either that no transfer is occurring, or that transfer is occurring but does not produce a depolarization because the dipoles are parallel.

Excitation Energy Transfer. The data in Figure 11 establishe that energy transfer occurs from the aromatic amino acids of liver alcohol dehydrogenase to bound auramine O. This transfer is reasonable because the absorption spectrum of bound auramine O has good overlap with the fluorescence emission of liver alcohol dehydrogenase giving a Förster overlap integral of 1.4×10^{-11} cm^6 mole^{-1}.

Figure 12 shows that the quenching of protein fluorescence is linearly proportional to the degree of enzyme saturation up to a $\bar{\nu}$ of 0.74, indicating that the quenching efficiency per auramine O is insensitive to the fraction of auramine O molecules which are bound in pairs. This fraction is 0.37 for the last point in the figure, so that any nonlinearity greater than about 5% would have shown up in the data. The extrapolation implies that when the enzyme is half-saturated, the protein fluorescence is 50% quenched, and that quenching becomes complete as full saturation is reached. An overlap of quenching domains would have caused the quenching effectiveness of the second dye molecule to bind to be less than that of the first. Thus the quenching domains of the two auramine O molecules are independent. All this quenching cannot be accounted for by the observed transfer since the transfer efficiency per auramine O from liver alcohol dehydrogenase is only 0.3 or less.

Properties of the Auramine O Binding Site. The uniqueness of the interaction of auramine O with liver alcohol dehydrogenase should be emphasized. Liver alcohol dehydrogenase is the only protein of many tested which forms a fluorescent complex with auramine O, or binds to auramine O as demonstrated by equilibrium dialysis. All of the evidence presented here is consistent with there being two identical noninteracting auramine O binding sites. What is the relation between these sites and the active sites of the enzyme? It is possible to account for the observations described in this paper on the basis of either auramine O sites adjacent to the active sites, or separate and distinct from the active sites. For the latter possibility it is necessary to postulate a dye-induced conformational change leading to a change in configuration at the active sites and a conformational change induced by coenzyme binding leading to modification of the dye binding regions. While Bränden (1965) has obtained X-ray diffraction evidence that conformational changes may take place in the presence of coenzyme and inhibitor, there is no direct evidence at present that they occur in solution under the conditions used for our experiments.

Either adjacent or separate sites could explain the noncompetitive inhibition by auramine O with respect to NAD^+ and ethanol as determined by kinetics, the increase in the auramine O association constant in the presence of NAD^+ as shown by kinetics and equilibrium dialysis, the quenching of auramine O fluorescence by NAD^+, and the release of auramine O from the enzyme by NAD^+ and pyrazole together. Further studies are underway to elucidate the position of the auramine O binding site relative to the enzyme active site.

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